PCT/EP 96 / 01706

The Patent Office

The Patent Office
Cardiff Road
Newport
Gwent
NP9 1RH

REC'D 10 JUN 1996
WIPO PCT

PRIORITY DOCUMENT

I, the undersigned, being an officer duly authorised in accordance with Section 62(3) of the Patents and Designs Act 1907, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

Signed

Dated 10 · 4 · 1996

For Official use

- 8 MAR 1996

Your Reference: **PT/pmh/P31408**

9604909.3

...e type, or write in dark ink using CAPITAL letters. A prescribed fee is payable for a request for grant of a patent. For details, please contact the Patent Office (telephone 071-483 4700).

Rule 16 of the Patents Rules 1990 is the main rule governing the completion and filing of this form.

❷ Do not give trading styles, for example, 'Trading as XYZ company', nationality or former names, for example, 'formerly (known as) ABC Ltd' as these are not required.

Warning

After an application for a Patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977 and will inform the applicant if such prohibition or restriction is necessary. Applicants resident in the United Kingdom are also reminded that under Section 23, applications may not be filed abroad without written permission unless an application has been filed not less than 6 weeks previously in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction revoked.

The Patent Office

Request for grant of a Patent

Form 1/77 **Patents Act 1977**

❶ Title of invention

1 Please give the title of the invention -- PHARMACEUTICALS

❷ Applicant's details

☐ **First or only applicant**

2a If you are applying as a corporate body please give:
Corporate Name SMITHKLINE BEECHAM PLC

Country *(and State of incorporation, if appropriate)* UNITED KINGDOM

2b If you are applying as an individual or one of a partnership please give in full:

Surname

Forenames

2c **In all cases,** please given the following details:

Address: NEW HORIZONS COURT
BRENTFORD
MIDDLESEX

UK postcode *(if applicable)* TW8 9EP

Country ENGLAND

ADP number *(if known)* 5820474003

2d, 2e and 2f: *If there are further applicants please provide details on a separate sheet of paper*

☐ **Second applicant** ***(if any)***

2d If you are applying as a corporate body please give:
Corporate Name

Country *(and State of Incorporation, if appropriate)*

2e If you are applying as an individual or one of a partnership please give in full:

Surname:

Forenames:

2f **In all cases,** please give the following details:

Address:

UK postcode *(if applicable)*
Country
ADP number *(if known)*

❸ *An address for service in the United Kingdom must be supplied*

Please mark correct box.

❸ **Address for service details**

3a Have you appointed an agent to deal with your application?

Yes ☒ No ☐ ➲ ***go to 3b***

❶ ***please give details below***

Agent's name: TOCHER, PAULINE
Agent's address: CORPORATE INTELLECTUAL PROPERTY
SMITHKLINE BEECHAM PLC
SB HOUSE
GREAT WEST ROAD
BRENTFORD
MIDDLESEX

Postcode: TW8 9BD
Agent's ADP number: 655674000 2

3b: *If you have appointed an agent, all correspondence concerning your application will be sent to the agent's United Kingdom address.*

3b If you have not appointed an agent please give a name and address in the United Kingdom to which all correspondence will be sent:

Name:

Address

Postcode

Daytime telephone number *(if available)*

ADP number *(if known)*

❹ Reference number PT/pmh/P31408

4. Agent's or applicant's reference number *(if applicable)*

❺ Claiming an earlier application date

5. Are you claiming that this application be treated as having been filed on the date of filing of an earlier application?

Please mark correct box

Yes ☐ No ☒ ➲ *go to 6*

please give details below

☐ number of earlier application or patent number

☐ filing date *(day month year)*

☐ and the Section of the Patents Act 1977 under which you are claiming:

Please mark correct box

15(4) (Divisional) ☐ 8(3) ☐ 12(6) ☐ 37(4) ☐

❻ Declaration of priority

If you are declaring priority from a PCT Application please enter 'PCT' as the country and enter the country code (for example, GB) as part of the application number

Please give the date in all number format, for example, 31/05/90 for 31 May 1990

6. If you are declaring priority from previous application(s), please give:

Country of Filing	Priority application number *(if known)*	Filing Date *(day, month, year)*

❼

Inventorship

- any applicant is not an inventor
- there is an inventor who is not
- an applicant, or
- any applicant is a corporate body.

7. Are you (the applicant or applicants) the sole inventor or the joint inventors?
Please mark correct box
Yes ☐ No ☒ ➲ *A Statement of Inventorship on Patents form 7/77 will need to be filed (see Rule 15).).*

❽ *Please supply duplicates of claim(s), abstract, description and drawings).*

❽ Checklist

8a Please fill in the number of sheets for each of the following types of document contained in this application

Continuation sheets for this Patents Form 1/77 ☐

Claim(s)	-	Description	28
Abstract	-	Drawing(s)	-

8b Which of the following documents also accompanies the application?

Please mark correct box(es)

Priority documents *(please state how many)* ☐

Translation(s) of Priority documents *(please state how many)* ☐

Patents Form 7/77 - Statement of Inventorship and Right to Grant ☐

Patents Form 9/77 - Preliminary Examination Report ☐

Patents Form 10/77 - Request for Substantive Examination ☐

❾ *You or your appointed agent (see Rule 90 of the Patents Rules 1990) must sign this request.*

❾ Request

I/We request the grant of a patent on the basis of this application.

Please sign here ➡

Signed P. Tocher Date: 7 March 1996
(day month year)

PAULINE TOCHER

A completed fee sheet should preferably accompany the fee.

Please return the completed form, attachments and duplicates where requested, together with the prescribed fee to either;

☐ ***The Comptroller***
The Patent Office
Cardiff Road
Newport
Gwent
NP9 1RH

or

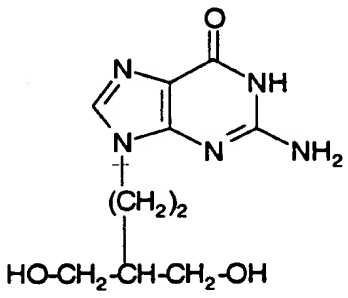
☐ ***The Comptroller***
The Patent Office
25 Southampton Buildings
London
WC2A 1AY

PHARMACEUTICALS

This invention relates to treatment of human immunodeficiency virus (HIV) and hepatitis B virus (HBV) infection.

When used herein, 'treatment' includes prophylaxis as appropriate.

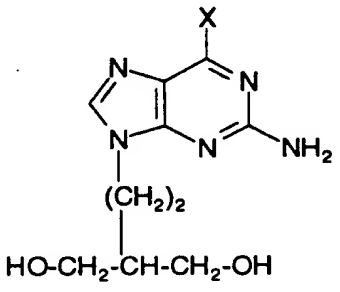
EP-A-141927 (Beecham Group p.l.c.) discloses penciclovir (PCV), the compound of formula (A):



(A)

and salts, phosphate esters and acyl derivatives thereof, as antiviral agents. The sodium salt hydrate of penciclovir is disclosed in EP-A-216459 (Beecham Group p.l.c.). Penciclovir and its antiviral activity is also disclosed in Abstract P.V11-5 p.193 of 'Abstracts of 14th Int. Congress of Microbiology', Manchester, England 7-13 September 1986 (Boyd et. al.).

Orally active bioprecursors of the compound of formula (A) are of formula (B):



(B)

and salts and derivatives thereof as defined under formula (A); wherein X is C_{1-6} alkoxy, NH_2 or hydrogen. The compounds of formula (B) wherein X is C_{1-6} alkoxy or NH_2 are disclosed in EP-A-141927 and the compounds of formula (B) wherein X is hydrogen, disclosed in EP-A-182024 (Beecham Group p.l.c.) are preferred prodrugs. A particularly preferred example of a compound of formula (B) is that wherein X is hydrogen and wherein the two OH groups are both in the form of the acetyl derivative, described in Example 2 of EP-A-182024, hereinafter referred to as famciclovir.

EP-A-388049 (Beecham Group p.l.c.), discloses the use of penciclovir/famciclovir in the treatment of hepatitis B virus infection.

The antiviral activity against hepatitis B virus appears to be dependent on intracellular formation of penciclovir triphosphate (PCV-TP). The HBV polymerase has many enzymatic activities, including formation of a covalent bond between the polymerase and dGMP, addition of T-A-A, followed by RNA-directed DNA polymerase (reverse transcriptase) and DNA-directed DNA synthesis.

The triphosphate derivative of penciclovir inhibits the RNA-directed DNA polymerase (reverse transcriptase) activity of human immunodeficiency virus type 1 (HIV-1). The reverse transcriptase of HIV-1 is a virus-encoded enzyme essential for the conversion of genomic RNA into proviral ds-DNA.

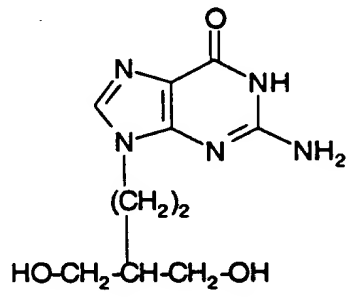
It has now been shown that the (R)-enantiomer of PCV-TP is more active than the (S)-enantiomer in respect of inhibition of HBV DNA polymerases and in respect of inhibition of HIV-1 reverse transcriptase.

Accordingly, the present invention provides a method of treatment of:

i) HIV-1 infections in mammals, including humans; or

ii) HBV infections in mammals, including humans;

which method comprises the administration to the human in need of such treatment, an effective amount of the (R)-enantiomer of the triphosphate of a compound of formula (A):



(A)

or a pharmaceutically acceptable salt thereof.

The (R)-PCV-TP is administered in the form of a compound which is a bioprecusor to allow absorption and penetration through the cell wall. Selectivity for the virus infected cell, especially HIV infected cells, can be achieved by selecting a bioprecursor which is activated preferentially by the virally encoded protease. The bioprecursor may be in the form of a derivative of (R)-PCV-MP which liberates intracellularly (R)-PCV-MP which is in turn converted to (R)-PCV-TP.

The compound may be administered by the oral route to humans and may be compounded in the form of syrup, tablets or capsule. When in the form of a tablet, any pharmaceutical carrier suitable for formulating such solid compositions may be used, for example magnesium stearate, starch, lactose, glucose, rice, flour and chalk. The compound may also be in the form of an ingestible capsule, for example of gelatin, to contain the compound, or in the form of a syrup, a solution or a suspension. Suitable liquid pharmaceutical carriers include ethyl alcohol, glycerine, saline and water to which flavouring or colouring agents may be added to form syrups. Sustained release formulations, for example tablets containing an enteric coating, are also envisaged.

For parenteral administration, fluid unit dose forms are prepared containing the compound and a sterile vehicle. The compound depending on the vehicle and the concentration, can be either suspended or dissolved. Parenteral solutions are normally prepared by dissolving the compound in a vehicle and filter sterilising before filling into a suitable vial or ampoule and sealing. Advantageously, adjuvants such as a local anaesthetic, preservatives and buffering agents are also dissolved in the vehicle. To enhance the stability, the composition can be frozen after filling into the vial and the water removed under vacuum.

Parenteral suspensions are prepared in substantially the same manner except that the compound is suspended in the vehicle instead of being dissolved and sterilised by exposure to ethylene oxide before suspending in the sterile vehicle. Advantageously, a surfactant or wetting agent is included in the composition to facilitate uniform distribution of the compound of the invention.

As is common practice, the compositions will usually be accompanied by written or printed directions for use in the medical treatment concerned.

A suitable dosage unit might contain from 50mg to 1g of active ingredient, for example 100 to 500mg. Such doses may be administered 1 to 4 times a day or more usually 2 or 3 times a day. The effective dose of compound will, in general, be in the range of from 0.2 to 40mg per kilogram of body weight per day or, more usually, 10 to 20 mg/kg per day.

The present invention also provides the use of the (R)-enantiomer of the triphosphate of a compound of formula (A) or a bioprecursor therefor, in the preparation of a medicament for use in the treatment of:

i) HIV-1 infections in mammals, including humans, which mammals are infected with herpesviruses; or

ii) HBV infections in mammals, including humans.

Such treatment may be carried out in the manner as hereinbefore described.

The present invention further provides a pharmaceutical composition for use in the treatment of:

I) HIV-1 infections in mammals, including humans; or

ii) HBV infections in mammals, including humans;

which comprises an effective amount of the (R)-enantiomer of the triphosphate of a compound of formula (A), or a bioprecursor therefor, and a pharmaceutically acceptable carrier.

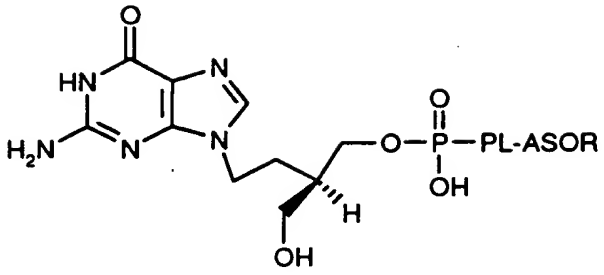
Such compositions may be prepared in the manner as hereinafter described.

The biological data describing the activity of (R)-PCV-TP is described by Shaw *et al*, Zoulim *et al* in 'Abstracts of the 35th Interscience Conference on Antimicrobial Agents and Chemotherapy' 17-20 September 1995, H13, p182 and H36, p191 and Schinazi *et al* in 'Antiviral Research' 1995, Supplement 1, 146, A304, and also in the attached draft manuscript by Zoulim and the attached extract from the poster presented in support of the Schinazi reference.

The following examples illustrate bioprecursors of the (R)-enantiomer of the triphosphate of a compound of formula (A). Examples 1, 2 and 5 are of potential interest in treatment of HBV and Examples 3 and 4 are of potential interest in treatment of HSV in HIV infected patients.

Example 1

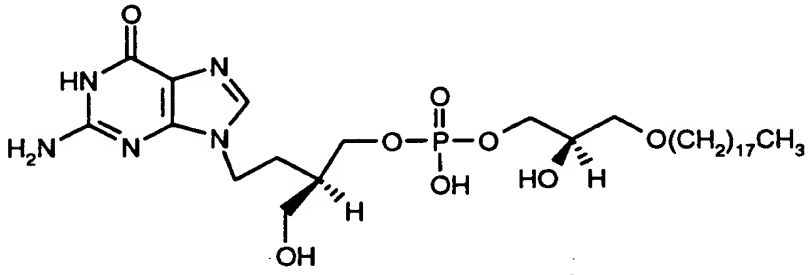
PL-ASOR derivative



The (S) enantiomer of a PCV derivative with a protecting group on one hydroxyl group is phosphorylated and the the protecting group is then removed to afford (R)-PCV-MP. (R)-PCV-MP is activated and coupled to PL-ASOR by a procedure such as that described in Drug Delivery 2, 136, 1995. The resulting conjugate may have multiple (R)-PCV-MP moieties per protein molecule.

Example 2

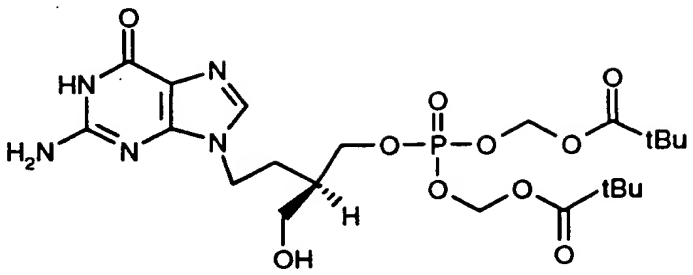
Phospholipid derivative



(R)-PCV-MP or a protected form thereof (prepared as in Example 1) is converted to this derivative by alkylation, or the phosphate group is activated and coupled to a protected form of the lipid, followed by deprotection.

Example 3

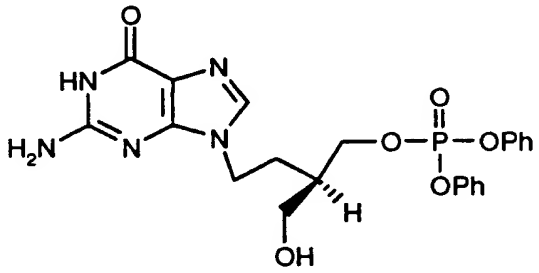
(R)-MP Bis(POM) derivative



(R)-PCV-MP or a protected form thereof (prepared as in Example 1) is converted to the bis(POM) derivative by alkylation with pivalyloxymethyl chloride by the procedure of J. Pharmaceutical Sci. 72, 324-5, 1983 or of J. Med. Chem. 38, 1372-9, 1995 and the optionally present protecting group is removed.

Example 4

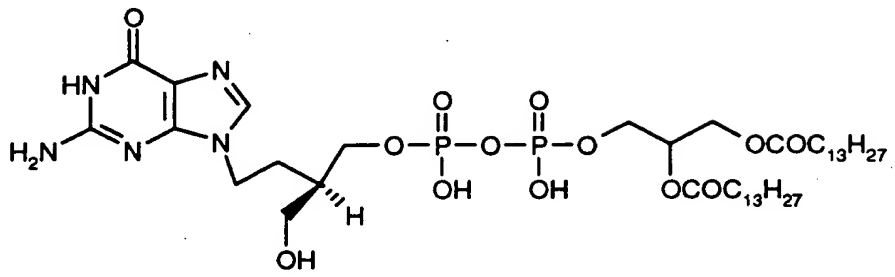
(R)-MP Diphenyl ester



The (S) enantiomer of a PCV derivative with a protecting group on one hydroxyl group is treated with diphenyl phosphorochloridate and the protecting group is removed.

Example 5

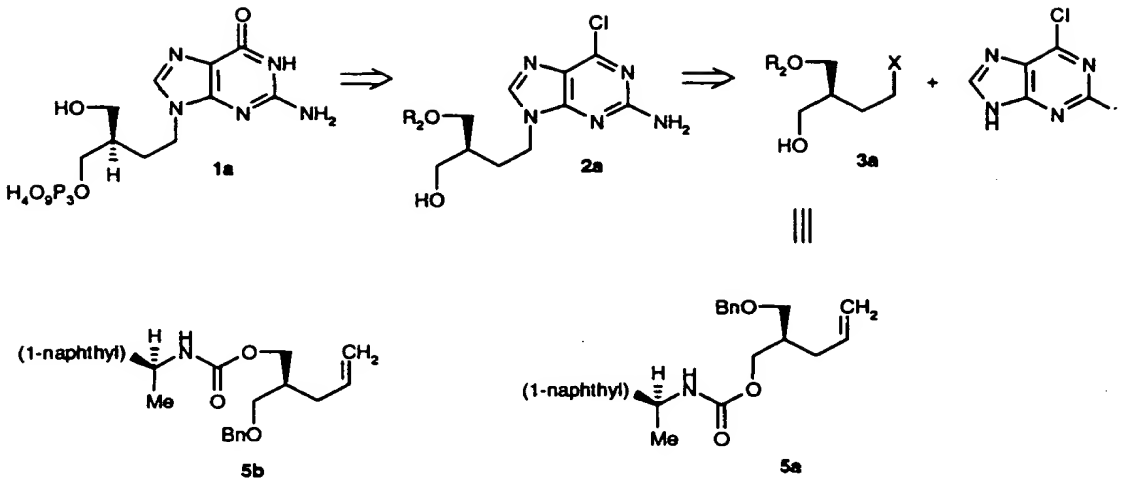
Dimyristoylglycerol diphosphate derivative



(R)-PCV-MP or a protected form thereof (prepared as in Example 1) is coupled with an activated form of *sn*-1,2-dimyristoylglycerol phosphate and the optionally present protecting group is removed.

Description - preparation of (R)-PCV phosphate

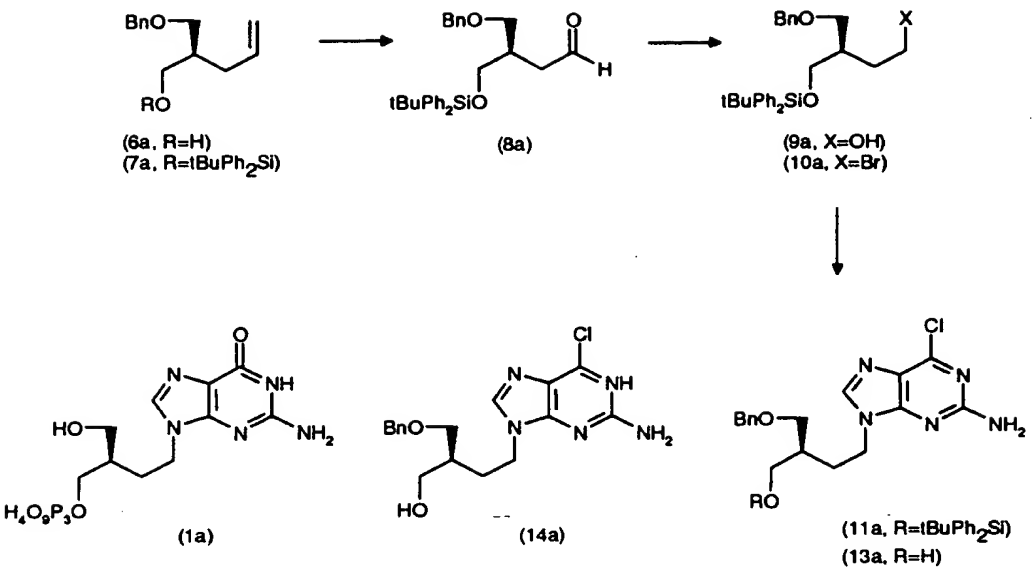
Retrosynthetic analysis of (R)-penciclovir triphosphate **1a** yields the mono-protected synthon **2a**, scheme 1. Further disconnection yields the chiral fragment **3a** and 2-amino-6-chloropurine **4**. The synthetic equivalent of the synthon **3a** is the urethane **5a** used by Kishi *et al* in the total synthesis of monensin[2]. By analogy for the synthesis of (S)-penciclovir triphosphate **1b** requires the urethane **5b** as starting material..The urethanes **5a** and **5b** have also been employed in the synthesis of (R)- and (S)-1-(3-hydroxymethylpyrrolidin-1-yl)cytosine[3].



Where: R_1 and R_2 are orthogonal protecting groups and X= good leaving group

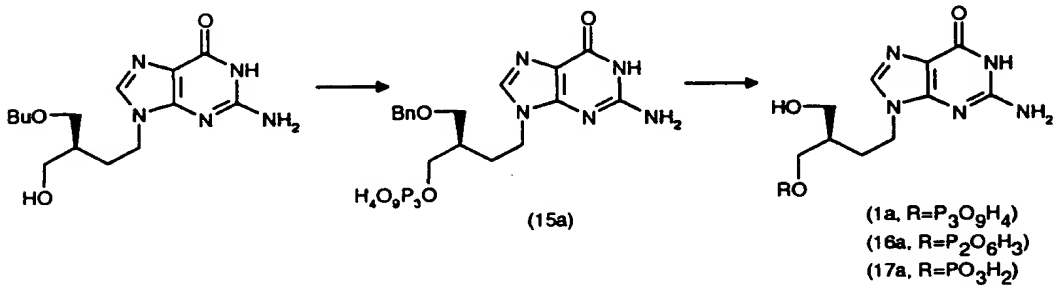
The diastereomeric urethanes **5a** and **5b** were separated by medium pressure column chromatography and were shown by analytical normal phase chromatography to be 86% d.e. in both cases. Reduction of the urethanes **5a** and **5b** with LAH gave the mono-benzyl ethers **6a** and **6b**[2]. The optical rotation of the levorotary mono-benzyl ether **6a** (α_D^{22} - 12.4° (c = 1.00, chloroform)); lit.[2] α_D^{22} - 12.1° (c = 0.68, chloroform)) had previously been established as possesing the **S** configuration. The mono-benzyl ethers **6a** and **6b** were silylated with the sterically bulky t-butyldiphenylsilyl group to give **7a** and **7b** in order to avoid possible silyl migration in later steps. The olefins **7a** and **7b** were firstly ozonised in good yield to the aldehydes **8a**(78%) and **8b**(91%) and then reduced to the alcohols **9a** and **9b** with Dibal-H.

For the synthesis of (R)-penciclovir triphosphate **1a** the alcohol **9a** was converted to the bromide **10a**(Br_2, PPh_3, DMF, 47%) in moderate yield. Alkylation of 2-amino-6-chloropurine **4** with the bromide **10a**,under literature conditions[4],

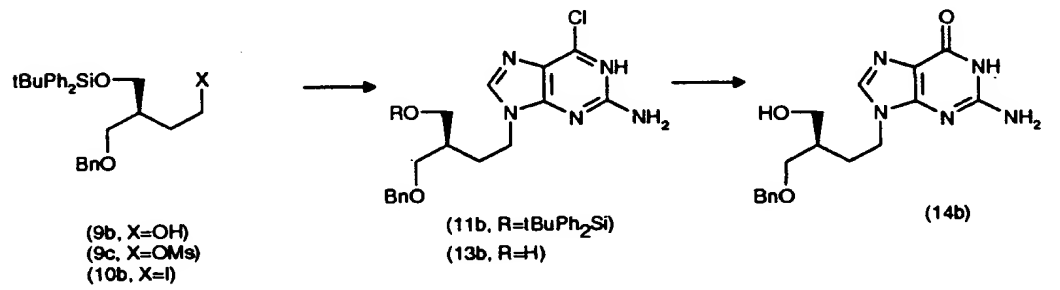


gave as the major product the N-9 alkylated nucleoside **11a** (75%) which was separated from the unwanted N-7 isomer **12a** (14%) by column chromatography. The regiochemistry of addition was simply confirmed from the undecoupled ^{13}C NMR spectrum of (**12a**)[5]. The optical purity of **11a** was not amenable to analysis by chiral HPLC. However, removal of the silyl protecting group of **11a** by acidic hydrolysis gave not only the chloropurine **13a** (42%, 93% e.e.) but also some of the desired guanosine nucleoside **14a** (23%,H94/0223% e.e.) both of which were amenable to chiral HPLC analysis. Prolonged acidic hydrolysis smoothly converted the chloropurine **13a** to the guanosine nucleoside **14a** (75%).The phosphitylating agent 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one (van Boom reagent) had previously been used for the preparation of nucleoside H-phosphonates as synthons in oligonucleotide synthesis[6]. Phosphitylation of the nucleoside **14a** (90% e.e.) was carried out using the van Boom reagent following conditions similar to that in the literature[8] for the synthesis of nucleoside triphosphates[7]. The phosphitylated intermediate was then reacted with tri-n-butylammonium pyrophosphate followed by oxidation with iodine to give the crude benzyl protected nucleotide triphosphate **15a**. Eventually, conditions that allowed the removal of the benzyl protecting group from **15a** and which avoided complete hydrolysis of the triphosphate group were found by using a transfer hydrogenation procedure. Fortuitously, the hydrogenolysis of the benzyl group from **15a** could be monitored by analytical anion exchange HPLC which yielded not only (R)-penciclovir triphosphate **1a**, but also the(R)-diphosphate

16a and the (R)-monophosphate **17a**. The latter two penciclovir nucleotides being formed as a result of hydrolysis of the triphosphate group under the transfer hydrogenation reaction conditions.



The synthesis of the (S)-triphosphate **1b** was carried out under essentially identical conditions to those described for the synthesis of the (R)-triphosphate **1a**, except that the alcohol **9b** was converted firstly to the mesylate **9c** and then to the iodide **10b**. Alkylation of 2-amino-6-chloropurine **4** with **10b** again yielded the N-9 adduct **11b** as the major product along with a small amount of the unwanted N-7 adduct adduct **12b**. Acidic hydrolysis of **11b** yielded a small amount of the 6-chloropurine **13b** (6%) but mainly the desired guanosine nucleoside **14b** (85%).



Phosphitylation of **14b** (90% e.e.) with the van Boom reagent, followed by reaction with tri-n-butylammonium pyrophosphate and then iodine gave the crude triphosphate **15b**. Again transfer hydrogenation of **15b** was monitored by analytical anion exchange HPLC to yield not only the desired (S)-penciclovir triphosphate **1b** but also the (S)-diphosphate **16b** and the (S)-monophosphate **17b**. The optical rotation results of all the (R)- and (S)-penciclovir phosphates are shown in Table 1.

For each enantiomeric pair the magnitude and sign of the optical rotations are essentially equal and opposite. This suggests that there has been little or no loss of chiral integrity from the precursors **14a** and **14b**, 90% e.e. in both cases, during the transfer hydrogenation conditions and therefore the optical purity of **1a**, **1b**, **16a**, **16b**, **17a** and **17b** to be approximately 90% e.e. in each case.

Method A:

Spherisorb silica (250 x 5.0 mm). Buffer A, Hexane; buffer B, hexane:methylene chloride (1:1); buffer C, hexane:ethanol (80:20). Eluant isocratic 40%A: 59%: 1%C, 1.00 ml/min. U.V. detection 220 n.m.

Retention time 5a (major 9.48 mins, minor 8.98 mins), 5b (major 9.23 mins, minor 9.69 mins)

Method B:

Merck RP select B (125 x 4 mm) uM. Buffer A, TFA (0.1%) in water; buffer B TFA (0.1%) in acetonitrile. Eluant, gradient running from 5% to 80%B over 40 minutes, then 10 minutes at 80%B. Flow 2.00 ml/min. U.V. detection at 215 n.m.

Method C:

Chiralpak AD (250 x 4.6 mm). Eluant isocratic hexane:ethanol (7:3), 1.00 ml/min. u.v. detection at 220 n.m.

Method D:

Chiralpak AD (250 x 4.6 mm). Eluant isocratic hexane:ethanol (1:1) containing 0.1% DEA, 1.0 ml/min. U.V. detection at 240 n.m.

Method E:

Chiracel OB (250 x 4.6 mm). Eluant isocratic hexane:ethanol (98:2), 1.00 ml/min. U.V. detection 220 n.m.

Method F:

Chiracel OC (250 x 4.6 mm) isocratic hexane:ethanol (95:5) containing 0.1% DEA, 1.00 ml/min.U.V. detection at 220 n.m.

Method G:

Rainin Hydropore SAX (100 x 4.6mm plus precolumn) 12μm . Buffer A, ammonium phosphate: methanol (9:1, 10mM, pH 5.7), buffer B, ammonium phosphate: methanol (9:1, 125mM, pH 5.7). Eluant gradient 0% to 100%B over 25minutes, 1.00ml/min. U.V. detection 254n.m.

2-Benzyloxymethyl-4-penten-1-ol (6)

To a stirred solution of sodium hydride (4.765g, 119.1 mmol) in dimethylformamide (50 ml) under argon with ice bath cooling was added a solution of 2-hydroxymethyl-4-penten-1-ol[8] (11.07g, 95.3 mmol) in dimethylformamide (75 ml) dropwise over 20 minutes. The cooling bath was removed and stirred for 1H before recooling the solution in an ice bath. A solution of benzylbromide (11.34 ml, 95.3 mmol) in dimethylformamide (75 ml) was added over 5 minutes, and after stirring for 18 hours at room temperature the reaction mixture was poured into brine (1.5L) and extracted with diethylether (4 x 300 ml). The organic fraction was washed with water (2 x 1.0L), dried over sodium sulphate, filtered and concentrated *in vacuo* to yield a light brown oil (17.40g). Silica gel crude oil (500 g) was purified by column chromatography using an increasing gradient from 10% diethylether: hexane 100% diethylether to yield ( ) (11.30g, 57.5%) as an oil; ^{1}H NMR (270 MHz, $CDCl_3$) δ 7.53 to 7.37 (5H, m) aromatic, 5.90 (1H, ddt), 5.15 (2H, m), 4.67 (1H, d), 4.62 (1H, d), 3.89 to 3.69 (3H, m), 3.62 (1H, dd), 2.38 (1H, bs), 2.23 (2H, ddd), 2.09 (1H, m);[13] (δ (67.8 MHz, $CDCl_3$) 138.00, 136.26, 128.48, 127.76, 127.63, 116.56, 73.47, 73.34, 65.73, 40.37, 32.79; m/z (CI) 207 (MH^+); $C_{13}H_{18}O_2$ requires C 75.69, H 8.80 found[c] 75.30, H 8.84.

(R)-(+)and (S)-(-)-2-benzyloxymethyl-4-penten-1-ol

To a stirred solution of (±)-2-benzyloxymethyl-4-penten-1-ol (8,52g, 41.3 mmol) in triethylamine (40ml,freshly distilled off P_2O_5) under nitrogen was added (R)-(-)-1-(1-naphthyl)ethylisocyanate (8.00g, 40.56 mmol). After 16 hours the

mixture was filtered and the solid washed with hexane. The filtrate was concentrated *in vacuo* to yield as a pale yellow 15.46g. Exhaustive column chromatography on silica gel eluting with methylene chloride: hexane: diethyl ether (10:10:1) gave the faster running diastereomer (5a) (5.15g, 30.9%) which was shown by analytical normal phase HPLC (Method A) to be 86% d.e. The slower running diastereomer (5b) (5.49g, 32.9%) was also shown to be 86% d.e. by HPLC (Method A). To a solution of the faster running diastereomer (5a) (5.15g, 12.76 mmol) in diethyl ether (250 ml) was added solid lithium aluminium hydride (484 mg, 12.76 mmol) and the mixture refluxed for 24H. The mixture was cooled in a water bath and water (0.43 ml) added, then a solution of sodium hydroxide (2.5M, 0.85 ml) and finally more water (1.06ml). After stirring at room temperature for 20 minutes the mixture was filtered through celite and the filtrate concentrated *in vacuo* to yield a clear oil (4.89g). Purification by column chromatography on silica gel eluting with a gradient from methylene chloride: hexane, diethyl ether (5:5:1) to (5:5:2) to yield (S)-(-)-2-benzyloxymethyl-4-penten-1-ol (6a) (1.97g, 75%). In a similar way the slower diastereomer (5b) (5.49g, 13.6 mmol) was reduced with lithium aluminium hydride to yield R-(+)-2-benzyloxymethyl-4-penten-1-ol (6b) (2.535g, 90.3%).

(S)-(-)-2-Benzyloxymethyl-4-penten-1-ol (6a)

Produced from lithium aluminium hydride reduction of (5a) faster running diasterethan on TLC in diethyl ether. [δ^{22}_{D}-12.44° (chloroform, c=1.00)], [lit α^{22}_{D}-12.1° (c=0.68, chloroform)]. Optical purity by chiral HPLC (Method E), retention time. Didn't work samples need to be repurified for accurate analysis.

(R)-(+)-2-Benzoxymethyl-4-penten-1-ol (6b)

Produced from lithium aluminium hydride reduction of (5b) slower running diastereothan TLC in diethyl ether. Optical purity by chiral HPLC (Method E) % retention time. See comments above.

(S)-2-benzyloxymethyl-1-O-(1-butyldiphenysilyl)-4-pentene (7b)

To a stirred solution of the (R)-(+)-alcohol (6b) (1.67g, % e.e, 8.1 mmol), under argon, in dimethylformamide (25 ml) was added imidazole (1.213g, 17.8 mmol) and then t-butyldiphenylchlorosilane (2.448g, 8.9 mmol). The mixture was stirred at RT for 16H and then poured into dilute brine and extracted with methylene chloride. The organic fraction was dried over sodium sulphate, filtered and

concentrated *in vacuo* to yield a colourless oil (4.18g). Column chromatography on silica gel eluting with 1% diethylether in hexane yielded (7b) (3.43g, 95%) as a clear oil. Forgot to take a copy of this NMR but simply remade.

(R)-2-Benzyloxymethyl-1-O-(t-butyldiphenylsilyl)-4-pentene (7a)

In a similar procedure to that described above the (S)-(-)-alcohol (6a) (1.71g, 8.90 mmol) was silylated which after column chromatography on silica gel yielded (7a) (2.07g, 83%) as a clear oil. ^{1}H NMR (200 MHz, $CDCl_3$) δ see above

(R)-2-Benzyloxymethyl-1-O-(t-butyldiphenylsilyl)butan-4-al (8a)

To a solution of the silyl protected olefin (1.32g, 2.99 mmol) (7a) in methylene chloride (60 ml) at -70°C was bubled ozone gas. After 10 minutes TLC indicated all starting material had been consumed and argon was bubbled through the solution until the exhaust gas was free of ozone. Then add a solution triphenylphosphine (0.94g, 3.59 mmol) in DCM (10 ml) was added and allowed to slowly warm to RT. After 16H the mixture was concentrated *in vacuo* to yield a pale yellow oil (3.606g). Chromatography on silica gel eluting with a gradient of 2% diethylether in hexane to 10% diethyl ether in hexane yielded the title compound (8a) (1.03g, 78%) as an oil. ^{1}H NMR (200 MHz, $CDCl_3$) δ9.75 (1H, t), 7.61 (4H, bd), 7.42 to 7.22 (11H, m), 4.45 (2H, s), 3.67 (2H, m), 3.55 (1H, dd), 3.45 (1H, dd), 2.52 (3H, bs), 1.03 (9H, s).

(S)-2-Benzyloxymethyl-1-O-(t-butylidiphenylsilyl)-butan-4-al (8b)

In a similar method to a solution of the olefin (7b) 3.315g, 7.46 mmol) in methylene chloride 150 ml under argon, internal temp -68°C was bubbled ozone gas. After 15 minutes solution went blue. Bubble argon until exhaust gas negative for ozone. After 30 mins add a solution of triphenylphosphine in DCM (30 ml) and allowed to slowly warm to RT and was stirred for 64H. Purified as above to yield (3.03g, 90%) as a clear oil.

(R)-2-Benzyloxymethyl-1-O-(t-butyldiphenylsilyl)-butan-1-ol (9a)

To a stirred solution of the aldehyde (8a) (2.132g, 4.80 mmol) in methylene chloride (50 ml) under argon at -70°C was added a solution of diisobutylaluminium hydride in methylene chloride (1.0M, 5.30 ml) dropwise over 5 minutes. After 1H at -70°C TLC indicated all starting material consumed. Ethyl aceate (1.77 ml) was then added, followed by water (1.6 ml) and the cooling bath removed and replaced with a

water bath. After 20 minutes was added sodium hydrogen carbonate (629 mg, 7.49 mmol) and the mixture stirred for 2.5 h. The solid was removed by filtration and washed with methylene chloride. The filtrate was concentrated *in vacuo* to yield as a clear oil (9a) (2.2g, 103%) which was used without further purification. ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.64 (4H, bm), 7.44 to 7.25 (11H, m), 4.48 (2H, s), 3.65 (4H, m), 3.60 (1H, dd), 3.45 (1H, dd), 2.73 (1H, bt), 2.02 (1H, m), 1.67 (2H, m), 1.04 (9H, s).

(S)-2-benzyloxymethyl-1-O-(t-butyldiphenylsilyl)-butan-1-ol (9b)

To a solution of the crude aldehyde in DCM (71 ml) at -68°C under argon was added dibal-H via syringe over 5 minutes. After 4.25h TLC indicated all starting material had been consumed and was quenched by addition of EtOAc (2.50 ml). The cooline bath was removed and replaced with a water bath and mixture stirred for a further 0.5H. Then solid sodium hydrogen carbonate (0.893g, 10.63 mmol) was added and stirred for a further 1H. The solid was removed by filtration and washed with methylene chloride to yield (9b) as a crude oil (2.89g, 95%) which was used without further purification.

(R)-2-Benzyloxymethyl-1-O-(t-butyldiphenylsilyl)-4-bromobutane (10a)

To a stirred solution of the alcohol (2.142g, 4.80 mmol) and triphenylphosphine (1.386, 5.29 mmol) in dimethylformamide was added bromine (0.272 ml, 5.29 mmol) dropwise. After stirring for 16H at RT the mixture was poured into water, the solid was filtered and washed with hexane. The aqueous fraction was washed with hexane and the combined organic fractions were dried over sodium sulphate and concentrated *in vacuo* to give a pale yellow oil (2.98g). Column chromatography on silica gel eluting with a gradient from 5% to 25% methylene chloride in hexane to yield the title compound (9a, 1.14g, 46.7%) as an oil. ^{1}H NMR (250 MHz$CDCl_3$) δ 7.65 (4H, m), 7.47 to 7.24 (11H, m), 4.48 (2H, s), 3.69 (2H, m), 3.53 (2H, m), 3.42 (2H, t), 2.12 to 1.92 (3H, m), 1.04 (9H, s).

(R)-2-benzyloxymethyl-1-O(t-butyldiphenylsilyl)-butan-4-O-methane sulphonate (9c)

To a stirred solution of the crude alcohol ( ) (2.894g, 6.45 mmol) in methylene chloride and triethylamine (1.35 ml, 9.68 mmol) under argon at -5°C was added methane sulphonyl chloride (0.6°ml, 7.74 mmol) dropwise over 7 minutes.

The mixture was warmed to 5°C, stirred for a further 1.5h, cooled to 0°C and dilute hydrochloric acid (1.25M) added. The organic phase was separated and the aqueous phase extracted with methylene chloride. The total organic fraction was washed with dilute sodium hydrogen carbonate solution, dried over sodium sulphate, filtered and evaporated *in vacuo* to yield a pale yellow oil (3.40g) which was used without further purification. ^{1}H NMR (200 MHz) δ 7.63 (4H, m), 7.45 to 7.23 (11H, m), 4.47 (2H, s), 4.26 (2H, dt), 3.68 (2H, m), 3.52 (2H, m), 2.88 (3H, s), 2.01 (1H, m), 1.88 (2H, m), 1.04 (9H, s).

(R)-2-benzyloxymethyl-1-O-(t-butyldiphenylsilyl)-4-iodo-butane (10b)

To a stirred solution of the mesylate (9c) (3.40g) (6.45 mmol) in acetone (60 ml), under nitrogen, was added sodium iodide (1.934g, 12.9 mmol). The mixture was then heated under reflux for 3.5h, cooled to RT and concentrated *in vacuo*. Methylene chloride and water were added, separated and the aqueous phase back extracted with methylene chloride. The organic phase was separated and the aqueous phase was washed first with sodium metabisulphite and then brine and dried over sodium sulphate. The solvent was removed *in vacuo* to yield a pale yellow oil (3.391g). Column chromatography on silica gel eluting with a gradient of 2% diethyl ether in hexane to 5% diethyl ether in hexane yielded the title compound (10b) (2.740g, 76%) as a clear oil. ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.63 (4H, m), 7.45 to 7.22 (11H, m), 4.47 (2H, s), 3.69 (2H, m), 3.54 (2H, m), 3.18 (2H, br), 1.97 (3H, m), 1.04 (9H, s).

(R)-2-benzyloxymethyl-1-O-(t-butyldiphenylsilyl)-4-(2-amino-6-chloropurin-9-yl)-butane (11a)

To a stirred solution of the bromide (10a) (1.017g, 2.00 mmol) in dimethylformamide (9.20 ml) and 2-amino-6-chloropurine (0.340g, 2.00 mmol) at RT under argon was added potassium carbonate (0.415g, 3.00 mmol). After 22H the reaction mixture was poured into water and extracted with methylene chloride:methanol (99:1). The combined organic extracts were washed with brine, dried over sodium sulphate, filtered and concentrated *in vacuo* to give a yellow oil (1.287g). Column chromatography on silica gel eluting with a gradient from 1% to 3% methanol in methylene chloride to yield (11a)as an oil (0.90g, 75.1%). ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.62 (5H, m), 7.48 to 7.22 (11H, m), 5.04 (2H, bs), 4.47 (2H, s),

4.10 (2H, t), 3.69 (2H, d), 3.48 (1H, dd), 3.37 (1H, dd), 2.09 to 1.78 (3H, m), 1.04 (9H, s).

(S)-2-benzyloxymethyl-1-O-(t-butyldiphenylsilyl)-4-(2-amino-6-chloropurin-9-yl)-butane (11b)

To a stirred solution of the iodide (10b) (2.40g, 4.90 mmol) in dimethylformamide (22.5 ml) was added 2-amino-6-chloropurine (834 mg, 4.91 mmol) and potassium carbonate (1.01g, 7.38 mmol) under argon at RT. After 30H was added methanol:methylene chloride (1:99, 250 ml) and the mixture poured into water (1L). The aqueous fraction was back extracted with methanol:methylene chloride (1:99, 3 x 250 ml). The total organic fraction was dried over sodium sulphate, filtered and concentrated *in vacuo* to give an oil (11.0g, containing residual dimethylformamide). The oil was taken up in methylene chloride (200 ml) and washed with water (1L). The aqueous phase was back extracted with methylene chloride (3 x 200 ml). The combined organic phase was again washed with water (1L), dried over sodium sulphate, filtered and concentrated *in vacuo* to give a yellow oil (3.162g). Column chromatography on silica gel eluting with DCM, methanol:methylene chloride (1:99) and finally methanol:methylene chloride (1.5:98.5) gave (11b) (2502g, 85%) as an oil containing ~5% DMF by ^{1}H NMR)

(R)-2-benzyloxymethyl-1-O-(t-butyldiphenylsilyl)-4-(2-amino-6-amino-7-yl)butane (12b)

The title compound was obtained as a later eluting fraction from the silica gel column purification of (11b). The resulting oil was recrystallised from ethyl acetate:hexane to give a white solid (457 mg, 15.6%) m.pt. 109-110°C, HPLC purity (Method) 98.0%. m/z 603 (M+H). ^{1}H NMR (400 MHz, DMSO-d6) 8.33 (1H, s), 7.54 (4H, m), 7.48 to 7.23 (11H, m), 6.61 (2H, bs), 4.42 (2H, t), 4.37 to 4.27 (2H, m), 3.64 (2H, m), 3.50 (2H, m), 1.95 (1H, m), 1.83 (2H, m), 0.93 (9H, s), ^{13}C (100.6 MHz, DMSO-d_6) 164.25, 159.84, 149.18, 1.42.03, 138.28, 134.91, 132.86, 132.82, 129.71, 128.10, 127.73, 127.25, 127.22, 114.61, 72.06, 69.42, 63.64, 44.51, 38.83, 29.92, 26.49, 18.65.

(S)-2-benzyloxymethyl-4-(2-amino-6-chloropurin-9-yl)-butan-1-ol (13a)

To a stirred solution of (11a) (158mg, 0.27 mmol) in tetrahydrofuran (4.0 ml) was added hydrochloric acid (2M, 4.0 ml) under argon at RT. After 24H excess

saturated sodium carbonate was added. The aqueous phase was extracted with ethyl acetate and then chloroform. The total organic fraction was dried over sodium sulphate, filtered and evaporated to give a clear oil (135 mg). Column chromatography on silica gel eluting with an increasing gradient of methanol 2% to 4% methanol in methylene chloride to yield (13a) (60 mg, 66%) as an oil. A sample was recrystallised from ethyl acetate:hexane to yield a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 (1H, s), 7.42 to 7.28 (5H, m), 5.11 (2H, bs), 4.50 (2H, dd), 4.17 (2H, m), 3.75 (2H, d), 3.56 (2H, m), 2.45 (1H, bs), 2.08 to 1.90 (2H, m), 1.83 (1H, m), m/z (FAB) 362 (M+H). Chiral HPLC (Method C) 93.6% e.e.

(S)-2-benzyloxymethyl-4-(9-guaninyl)-butan-1-ol (14a)

To a stirred solution of the chloropurine (13a) (325 mg, 0.9 mmol) in tetrahydrofuran (15 ml) was added hydrochloric acid (2M, 15 ml) and stirred at 60°C under argon, for 26H. The reaction mixture was cooled in a water bath and sodium hydroxide solution (10M) added until pH9. The aqueous phase was extracted with methanol:ethyl acetate (7:93, 4x35 ml). The organic phase was dried over sodium sulphate, filtered and evaporated *in vacuo* to give an oil (150 mg). On standing a white solid crystallised from the aqueous phase which was filtered off, washed with water (5 ml) and diethylether and dried *in vacuo* to give the title compound (14a) (175 mg, 56.8%). ^{1}H NMR (400 MHz, DMSO-d_6) 10.55 (1H, s), 7.66 (1H, s), 7.40 to 7.22 (5H, m), 6.44 (2H, bs), 4.51 (1H, t), 4.42 (2H, t), 4.00 (2H, t), 3.49 to 3.30 (4H, m), 1.76 (2H, q), 1.64 (1H, m). HPLC purity (Method) 98.3%, chiral HPLC (Method D) 89.6% e.e., retention time major peak 10.3 mins, minor peak 6.9 minutes. ($C_{17}H_{21}N_5O_3$,-7.7ppm, NH_3Cl) m/z 343.1617. The oil was purified by column chromatography on silica gel eluting with an increasing gradient of methanol (8-22%) in methylene chloride to yield (14a) (87 mg, 28.2%) as an oil.

(R)-2-benzyloxymethyl-4-(2-amino-6-chloropurin-9-yl)-butan-1-ol (13b)

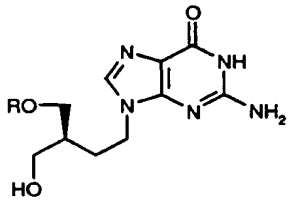
To a stirred solution of (11b) (248mg, 0.42 mmol) in THF (6.22 ml), at RT under argon, was added hydrochloric acid (2.0M, 6.2 ml). After 26H the tetrahydrofuran was removed *in vacuo*. The aqueous phase was extracted with ethyl acetate (3 x 30 ml), and the total organic fraction dried over sodium sulphate, filtered and evaporated *in vacuo* to give an oil. Column chromatography on silica gel eluting with chloroform and then an increasing gradient of methanol (1 to 4%) gave (13b)

(96mg, 63.8%). The resulting oil was recrystallised from ethyl acetate:hexane to give a white solid (86 mg, 57.2%). Chiral HPLC (Method C) 86.2% e.e.

(R)-2-benzyloxymethyl-4-(9-guaninyl)-butan-1-ol (14b)

To a stirred solution of the chloropurine (11b) (2.235g, 3.71 mmol) in tetrahydrofuran (56 ml), under argon at RT, was added hydrochloric acid (2.0M, 56 ml). The mixture was heated in the region 65-70°C for 20 hours, cooled to RT and neutralised with sodium hydroxide (12.5M, ca 9 ml) until pH7. The tetrahydrofuran was removed by evaporation *in vacuo*. The aqueous phase was extracted with methylene chloride (100 ml) producing an emulsion and solid was removed by filtration. The solid was washed with methylene chloride (100 ml) and diethyl ether (50 ml). The aqueous phase was back extracted with methylene chloride (100 ml, from solid wash). The combined organic extracts were dried over sodium sulphate, filtered, and concentrated *in vacuo* to give an oil (1.616g). The aqueous phase was readjusted to pH 7 and cooled in an ice bath for 2H. The resulting solid was washed with water and diethyl ether and combined with the first crop. The combined solid were taken up in methanol, filtered and concentrated *in vacuo* to give (14b) (944 mg, 74.5%). HPLC purity 98.2%, chiral HPLC (Method D), 81.4% e.e.. A sample (755 mg) was recrystallised from water (ca 90 ml) and allowed to slowly cool to RT. The resulting solid was filtered, washed with water and dried *in vacuo* to give (14b) (609 mg, 80.7% recovery). Chiral HPLC (Method D) 89.9% e.e. retention time major peak 6.7 mins, minor peak 10.4 mins. HPLC purity (Method B) 98.9%.

$C_{17}H_{21}N_5O_3(H_2O)_{0.6}$ requires C = 57.43; H = 6.29 and N = 19.70 found C = 57.49, H = 6.07 and N = 19.72.



(1b, R=$P_3O_9H_4$)

(16b, R=$P_2O_6H_3$)

(17b, R=PO_3H_2)

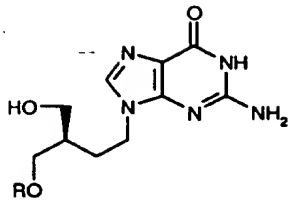
(S)-Penciclovir triphosphate (1b), diphosphate (16b) and monophosphate (17b)

A suspension of the (R)-nucleoside (14b) (174 mg, 0.51 mmol) was azeotroped with pyridine (2 x 10 ml). To a stirred suspension of ( ) in pyridine (1.22 ml) and dimethylformamide (4.62 ml) was added a solution of 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one (113 mg, 0.57 mmol) in dioxane (1.13 ml, plus 0.5 ml wash). The nucleoside immediately started to dissolve and a slight yellow colour was observed. After 10 minutes a solution of tri-n-butylamine pyrophosphate (336 mg) in tri-n-butylamine (0.58 ml) and dimethylformamide (2.9 ml) was added. After a further 10 minutes a solution of iodine (1% w:v) in pyridine:water (98:2;v:v; 10.2 ml) was added. Fifteen minutes after the addition of the iodine the reaction was poured into water (80 ml) and extracted with chloroform (4 x 80 ml). The aqueous phase was concentrated *in vacuo* to give the crude benzyl protected triphosphate (15b) (460 mg). To a soution of the crude triphosphate ( ) (460 mg) in water:methanol (1:1, 80 ml) was added palladium on carbon catalyst (Johnson-Matthey, Type 487L, 174 mg) with stirring under argon. After 15 minutes the ctalyst was removed by filtration on celite and washed with not methanol:water (1:1, 90ml). To the filtrate was added ammonium formate (1.0g) and palladium catalyst (203 mg), with stirring under argon. The mixture was heated at reflux, both temperature 93-103°C, for 6 H. At which time the reaction had only proceeded ~50% to completion as judged by anion exchange HPLC (method). The hot reaction mixture was filtered through celite and the catalyst washed with hot water:methanol (1:1, 200 ml). The filtrate was concentrated *in vacuo* and lyopholysed from water (700 ml) to give a white solid (620 mg). This solid was re-submitted to the transfer hydrogenation conditions. To a solution of the crude partially deprotected triphosphate (620 mg). This solid was re-submitted to the transfer hydrogenation conditions. To a solution of the crude partially deprotected triphosphate (620 mg) in water:methanol (1:1, 160 ml) was added ammonium formate (480 mg) and palladium catalyst (50 mg), with stirring under argon. The mixture was heated, oil bath temperature 80°C, for 1.5H after which time the deprotection had deemed to have gone to completion, $\leq$ 1% ( ) remaining, as judged by anion exchange HPLC. The hot reaction mixture was filtered through celite and the catalyst washed with hot water:methanol (1:1, 200 ml). The filtrate was concentrated *in vacuo* to give a white solid (900 mg) which was

lyopholysed from water (1x500 ml and 1x150 ml) to give crude (1b), (16b) and (17b) (367 mg).

Half of this was purified to give:

50 mg PCV.TP
40 mg PCB.DP
10 mg PCV.MP



(1a, R=$P_3O_9H_4$)
(16a, R=$P_2O_6H_3$)
(17a, R=PO_3H_2)

(R)-Penciclovir triphosphate (1a), diphosphate (16a) and monophosphate (17a)

The (S)-nucleoside (14a) (174 mg, 0.51 mmol) was phosphenylated and converted to the crude benzyl protected (15a) under identical conditions to those described for the synthesis of its enantiomer (15b) above. The crude benzylated triphosphate (15a) was taken up in water:methanol (1:1, 80 ml) and stirred with palladium on carbon catalyst (Type 487L, 175 mg). After 20 minutes the mixture was filtered through celite and the catalyst washed with water:methanol (1:1, 80 ml). The filtrate was used for the transfer hydrogenation debenzylation reaction. To the filtrate (160 ml) was added ammonium formate (530 mg) and palladium on carbon catalyst (153 mg) and stirred under argon. The mixture was heated, oil bath temperature 76-88°C, and monitored by anion exchange HPLC (Method). After 3.5H palladium catalyst (305 mg) was added and after 5.5H extra palladium catalyst (142 mg) was added. After 6.5H the reaction was filtered through celite and the catalyst washed with hot water:methanol (1:1, 200 ml) and the filtrate concentrated *in vacuo*. The filtrate was dissolved in water:methanol (1:1, 160 ml), ammonium

formate (265 mg) and palladium catalyst (150 mg). The mixture was stirred under argon and heated in an oil bath in the region 76-80°C. After 5.5H the deprotection had deemed to have gone to completion by anion exchange HPLC, ≤ 1% ( ) remaining. The hot reaction mixture was filtered through celite and the catalyst washed with hot methanol:water (1:1, 100 ml). The filtrate was concentrated *in vacuo*, azeotroped with water (25 ml) to give an oil (830 mg). Then lyopholysed from water (1 x 300 ml and 1 x 125 ml) to give a white solid (376 mg).

References

1. Sime, J.T., Barnes, R.D., Elson, S.W., Jarvest, R.L. and O'Toole, K.J., *J. Chem. Soc. Perkin Trans. 1*, 1992, 1653-1658; Hannah, J., Tolman, R.L., Karkas, J.D. Liou, R., Perry, H.C. and Field, A.K., *J. Heterocyclic Chem.*, 1989, **26**, 1261-1276.
2. Furuyama, T., Wang, C.-L.J. and Kishi, *J. Am. Chem. Soc.*, 1979, **101**, 260-262.
3. Harnden, M.R. and Jarvest, R.L., *J. Chem. Soc. Perkin Trans 1*, 1991, 2071-2079.
4. Harnden, M.R., Jarvest, R.L., Bacon, T.H. and Boyd, M.R., *J. Med. Chem.*, 1987, **30**, 1636-1642; Geen, G.R., Grinter, T.J., Kincey, P.M. and Jarvest, R.L., *Tetrahedron*, 1990, **46**, 6903-6914.
5. In the undercoupled ^{13}C NMR of ( ) the purine C-5 (114.53 ppm) is coupled to purine H-8 and to the methylene group (C-4) of the butylside change and appears as a quartet. Where as the purine C-4 (164.12 ppm) appears as a doublet as it is only coupled to the proton on C-8 of the purine.
6. Marugg, J.E., Tromp, M., Kuyl-Yeheskiel, E., van der Marel, G.A. and Van Boom, J.H., *Tetrahedron Lett.*, 1986, **27**, 2661-2664.
7. Ludwig, J. and Eckstein, F., *J. Org. Chem.*, 1989, **54**, 631-635.
8. Wasson, B.K., Gleason, C.H., Levi, I, Parker, J.M., Thompson, L.M. and Yates, C.H., *Can. J. Chem.* 1961, **39**, 923-932.

WHAT IS CLAIMED IS:

The subject matter of the invention described herein in all aspects and embodiments.

Taken from draft manuscript by Zoulim.

III. RESULTS

1) An in vitro assay for the expression of an enzymatically active DHBV reverse transcriptase

We had previously shown that the DHBV polymerase expressed in vitro in a reticulocyte lysate is enzymatically active if the template for the initiation of RT, ie ε, is provided during the translation of the viral enzyme. In figure 2, we show that, when the RNA sequence A which contains ε, DR1 and the 5' flanking region, is coexpressed in trans, the efficiency of reverse transcription was enhanced by approximately 50%. A similar increase of enzymatic activity was observed on both the priming reaction and DNA chain elongation.

2) Inhibitory effect of pyrophosphate analogs on the DHBV reverse transcriptase

Pyrophosphate analogs have been shown to be potent inhibitors of DNA polymerases as well as of reverse transcriptases (1). We have studied the inhibitory effect of PAA (phosphono acetic acid) and that of PFA (phosphonoformic acid) on the DHBV reverse transcription. As shown in figure 3, PAA did not inhibit minus strand DNA synthesis even at high concentration (1mM). By contrast, PFA showed a very potent inhibitory effect on DNA chain elongation but not on the priming reaction, at a concentration of 1 mM. This PFA concentration has been previously used to block DHBV reverse transcription in tissue culture cells (4).

3) Inhibitory effect of dGTP analogs on the elongation of minus strand DNA (reverse transcription)

We have compared the inhibitory activity of dGTP analogs on the elongation of minus strand DNA (see figure 4). Extended DNA chain is covalently linked to the viral polymerase. This allows for its study through 0.1% SDS-10% polyacrylamide gels. Viral DNA synthesis was

analyzed by the incorporation of dNTPs (dATP, TTP, dCTP) and radiolabelled α-^{32}P-dGTP. The level of α-^{32}P-dGTP incorporation was also measured by a dot assay on DE-81 filters. As shown in figure 4, the incorporation of α-^{32}P-dGTP in the presence of increasing concentrations of ACV-TP, R-PCV-TP, S-PCV-TP, ddG-TP and CDG-TP was reproducibly inhibited. At a concentration of 100 µM, ddG-TP, ACV-TP and R-PCV-TP showed an almost complete inhibition effect (> 75 %). S-PCV-TP was the less active compound and very high concentrations (1 mM) were needed to achieve a significant inhibition of reverse transcription (> 75 %). CDG was the most active compound since the same order of inhibition could be obtained at a concentration of 10 µM. The IC50 of ACV-TP, R-PCV-TP, ddG and CDG-TP was approximately 7 uM, 8 uM, 20 uM and < 1 uM , respectively. CDG was the most efficient compound followed by ACV and PCV. **R-PCV was more efficient than S-PCV.**

4) Effect of dGTP analogs on the incorporation of the first nucleotide of minus strand DNA, dGTP

In this experiment, we tested the inhibitory effect of this different analogs on DNA-priming (ie, incorporation of the first nucleotide of minus strand DNA, dGTP). The DHBV polymerase was incubated only with 32P-dGTP (0.15 µM final concentration, 3000 Ci/mmole). ACV, R-PCV, S-PCV, ddG and CDG were tested in the same range of concentrations. Results were in agreement with that obtained with viral DNA chain extension (figure 5).

ACV and R-PCV did inhibit the incorporation of the first nucleotide, dGTP. The IC50 of these two compounds was approximately 20 uM which was higher than that obtained with the DNA chain elongation. **Again R-PCV was more effective on the priming reaction than S-PCV which had an IC50 higher than 1 mM.** ddG was more efficient on the priming reaction than on DNA chain elongation since the IC50 for the priming reaction was approximately 1 uM. CDG was the most potent inhibitor of dGTP incorporation, since the inhibition was almost complete at a concentration of 10 µM.

ICAR Poster by R. Schinazi.

Inhibition of Viral Enzymes by PCVTP

Enzymic studies showed that racemic PCVTP was an efficient inhibitor of HIV-1 reverse transcriptase (RT) and that (*R*)-PCVTP was at least 20-fold more potent than the corresponding (*S*)-enantiomer using an RNA-dependent template with HIV RT (Figure 2 and Table 1). Using a DNA template, the enantiomers were essentially inactive when tested up to 10 μM. In contrast, using an M13mp18(+) strand DNA template, chain termination was observed at 10 μM. Both the (*R*)- and (*S*)-PCVTP inhibited DNA elongation, but it appears that the (*R*)-enantiomer is less selective (in addition to stops at G bases, stops at other bases were observed). Of interest was the finding of more G stops in a 130 base stretch with either PCVTP enantiomers and ddGTP than with ACVTP. As anticipated, chain termination only occurred at G bases with both ACVTP and ddGTP.

Table 1. Summary of Inhibition of Viral Enzymes by Penciclovir Triphosphate

Compound	Recombinant HSV-1[a] Activated DNA $IC_{50} \pm$ S.D., µM	HSV-2/Cell Derived[b] Activated DNA $IC_{50} \pm$ S.D., µM	Recombinant p66/51 HIV-1 RT rCdG[c] $IC_{50} \pm$ S.D., µM	Recombinant p66/51 HIV-1 RT dCdG[d] $IC_{50} \pm$ S.D., µM
Racemic PCVTP	15.5	26.7	2.7 ±1.6	> 10
(*R*)-PCVTP			0.91 ± 0.76	> 10
(*S*)-PCVTP			8.3 ± 4.5	> 10
(*S*)-PCVTP HSV-infected cell derived	54.2	23.6	> 10	ND
ACVTP	0.14 ± 0.11	0.051	0.060 ± 0.026	1.7
ddGTP	51.5 ± 31.9	ND	0.025 ± 0.037	0.021
PFA	8.9	ND	9.4 ± 3.2	ND

[a] 75 µl reaction mixture (1 unit enzyme, 50 mM Tris, pH 8.0, 150 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mg/ml BSA, 0.1 mM each dATP, dCTP, dTTP, 0.2 mg/ml activated DNA, 1 µM ^{3}H-dGTP, 8 Ci/mmol) incubated 60 min. at 37°C, stopped with 5% TCA/0.05% sodium pyrophosphate, harvested onto glass fiber sheets using Packard Filtermate 196 harvester and counted using Packard Matrix 9600 direct beta counter.

[b] Methodology as in *a*, except ^{3}H-dGTP activity: 42 Ci/mmole.

[c] Methodology as in *a*, except 100 µl reaction mixture: 1 unit enzyme, 100 µM Tris pH 8.0, 50 mM KCl, 2 mM $MgCl_2$, 5 mM DTT, 0.05 U/ml $(rA)_n.(dT)_{12-18}$, 1 µM ^{3}H-dGTP, 8 Ci/mmole

[d] Methodology as in *c*, except template-primer: 0.05 U/ml $(dC)_n.(dG)_{12-18}$